# The Fractionation of Trypsin by Electrophoresis in Starch Columns*)

Previous work done in this laboratory [1] showed that crystallized trypsin could be fractionated by paper electrophoresis at pH 2.7 in three active components. Further investigation [2] demonstrated that the main fraction of crystalline trypsin, obtained by paper electrophoresis, was not homogeneous when analyzed by free boundary electrophoresis at pH 5 in the presence of $Ca^{++}$.

The present work describes the fractionation of trypsin by electrophoresis in starch columns. This study was initiated in order to develop a preparative method for the obtention of trypsin fractions to be used for their structural and enzymatic characterization. Using starch columns buffered at pH 2.7 it was possible to demonstrate the appearance of three active components showing different specific activity against α-benzoyl-L-arginine-amide (BAA). The electrophoretic fractionation diagram obtained by this method was found to be quantitatively different of that obtained by paper electrophoresis at the same pH [1]. The analysis of a sample from another commercial source however showed the presence of only two main fractions.

The samples of trypsin were purchased from the *Worthington Biochemical Corporation* (Cryst. Trypsin, Lyophilized, lot TL 747–48) Freehold, N. Jersey, U.S.A. and from *Novo Industri* (Cryst. Trypsin Novo, batch 114–3).

Commercial potato starch washed with distilled water until the absorbance of the supernatant, at 280 mμ, was less than 0.02 units was used to pack the columns.

Sufficient amount of a slurry obtained by mixing one part of washed starch with four parts of buffer was poured into a glass tube of 3 × 80 cm until a 70 cm column of starch was obtained. The elctrophoresis were performed in an apparatus similar to that of KUNKEL [3].

The buffer used in all experiments was a mixture of formic acid-pyridine-water (1.125:0.25:98.625) of pH 2.7. A solution of 10 mg of trypsin in 0.5 ml of the buffer was applied to the top of the column and then washed down with 10 ml of the same buffer. Current was applied (350 volts, 8 mA) and the experiment allowed to run for 50 hours at room temperature. In these conditions, the temperature of the column during electrophoresis was about 30°C. After electrophoresis the column was mounted on a drop counting fraction collector and eluated with the pyridine-formic acid buffer at the rate of twelve 3 ml fractions per hour. Electrophoresis of the isolated trypsin fractions were obtained by the same general method.

*) This work has been supported in part by grants from the *Foreign Research and Technical Programs Division, Agricultural Research Service* of the *U. S. Department of Agriculture* (FG-Br-100) and the *Conselho Nacional de Pesquisas*.

Protein concentration was estimated by measuring the absorbance of the fractions, at 280 mμ, in a Beckman DU Spectrophotometer using cells of 1 cm optical path. The average recovery of trypsin applied to the column amounted to 96%. The proteolytic activity was determined by the method of CHARNEY AND TOMARELLI [4]. The hydrolysis of α-benzoyl-L-arginine amide was followed in a Technicon Autoanalyzer by measuring the colour formed by alkaline phenol and hypochlorite with the ammonea liberated by the trypsin [5].

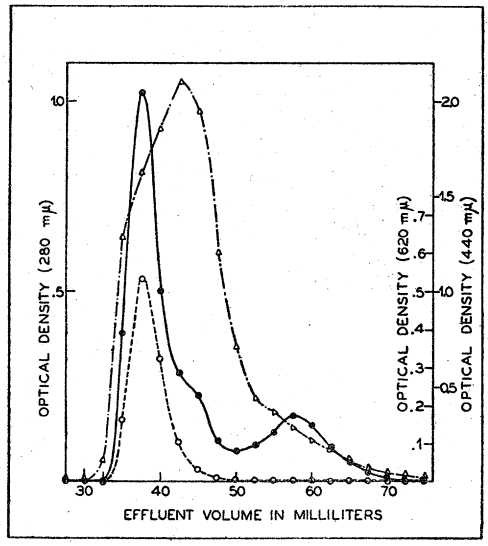


Fig. 1 — Column electrophoresis of the sample of Worthington trypsin

—●— protein value
-•-△-•- proteolytic activity
--○-- amidasic activity

In the experiments in which the electrophoresis was performed in the presence of $Ca^{++}$, sufficient $CaCl_2$ was added to the pyridine-formic acid buffer to make it 0.03M in respect to the salt. This $CaCl_2$ containing buffer was also used in this case to prepare the 2% trypsin solution to be applied to the column.

A typical result of the fractionation of Worthington Trypsin in starch columns is shown in Figure 1. Three different active fractions are separated. The separation of $F_1$ (the faster fraction) from $F_2$ (the fraction of intermediate mobility) is not complete.

A noteworthy feature of this diagram is the fact that the maximum of the proteolytic activity curve is not coincident with the maximum of the curve showing protein content. The maximum of activity appears to correspond to the $F_2$ fraction which is present in about one third of the $F_1$ fraction.

The specific activity against BAA of the three separated fractions and their relative amount are presented in TABLE I.

TABLE I

| | WORTHINGTON TRYPSIN | | NOVO TRYPSIN | |
|---|---|---|---|---|
| | % of total protein | Specific activity (1) | % of total protein | Specific activity (1) |
| Unfractionated trypsin | 100 | 0,65 | 100 | 1,01 |
| Fraction 1 | 53 | 0,95 | 73 | 1,23 |
| Fraction 2 | 22 | 0,76 | | |
| Fraction 3 | 25 | 0,25 | 27 | 0,72 |

(1) The specific activity is given as the slope, multiplied by $10^3$, of the straight line obtained by plotting the optical density at 620mμ against trypsin concentration in micrograms per milliliters.

An aliquot of the content of the tube which gave the highest reading for fraction I in the experiment shown in Figure 1 was again fractionated in identical conditions used for the electrophoresis of the whole trypsin (Fig. 2). The results of these experiments tend to demonstrate that no further fractionation occurred or degradation of trypsin took place in the conditions of the experiment. The curve representing proteolytic activity of $F_1$ shows a slight tailing which is probably due to contamination of the main component ($F_1$) with the fraction of intermediate mobility ($F_2$) which apparently showed the highest activity against azo-casein.

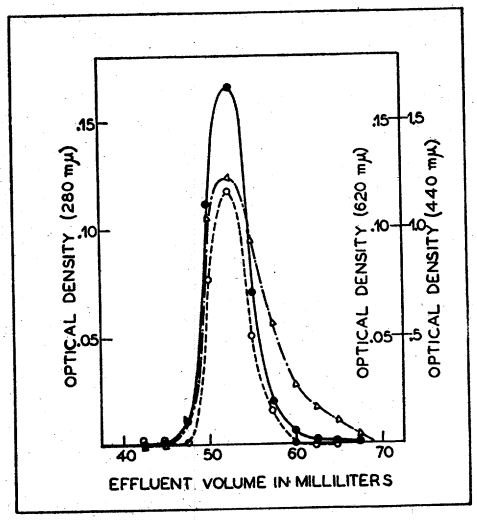


Fig. 2 — Re-electrophoresis of the main fraction obtained from the Worthington trypsin

—●— protein value
-·-△-·- proteolytic activity
--○-- amidasic activity

These conclusions are also corroborated by the experiments in which trypsin was fractionated in the presence of stabilizing concentration of calcium (0.03M $CaCl_2$). The electrophoretic pattern obtained in this case is presented in Figure 3 and they also show the appearance of three main fractions. The separation between $F_1$ and $F_2$ in this case was even less complete. However, the presence of $F_2$ with an apparently higher proteolytic activity than $F_1$ is clearly indicated by the position of the activity maximum which is shifted from the protein maximum.

Previous experiments in this laboratory by using free electrophoresis at pH 5, in the presence of $Ca^{++}$ have shown that the main trypsin fraction obtained by paper electrophoresis at pH 2.7 can be separated in two fractions [2]. The faster fraction in this case amounted to 56% and the slower one to 26% of the total protein. These data are in good agreement with the results obtained in the present experiment, i.e., the main trypsin peak is partially separated in two components amounting respectively to 55% and 21% of the whole trypsin.

The electrophoretic analysis of the Novo Trypsin gave, however, a somewhat different fractionation diagram (Figure 4). In the samples of trypsin from this source which we analyzed only two fractions were obtained. The fraction of intermediate mobility being aparently absent and the activity maximum is not shifted from the protein maximum. The specific activity of the fractions are also different and are given in TABLE I.

LIENER [6] using chromatography in carboxy methyl cellulose has also shown the existence of three active components in crystalline trypsin. This author, believes

that the minor active components may represent the products of secondary reactions involving cleavage sites, different of that studied by DAVIS & NEURATH [7].

GLADNER AND FOLK [8] have shown that carboxy-peptidase B releases from many commercial trypsin preparations about one equivalent of C-terminal lysine. On the other hand no lysine was obtained by incubation of trypsinogen activation mixture with the same enzyme.

The results of GLADNER AND FOLK [8], and the presence in crystalline trypsin of active fractions with different activities appear to lend support to Liener's suggestion.

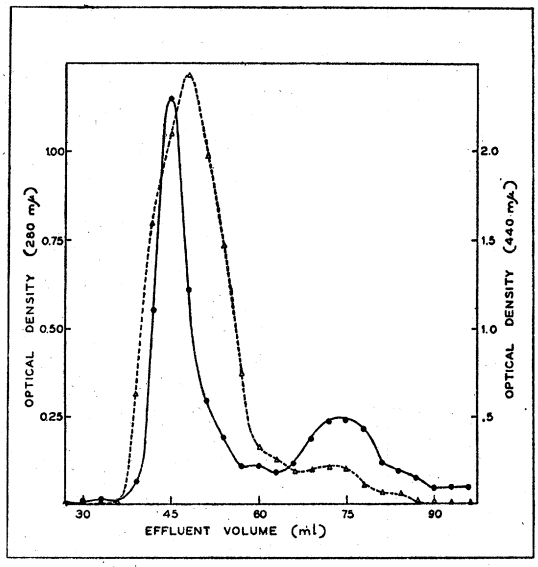


Fig. 3 — Column electrophoresis of the Worthington trypsin in presence of 0.03M $CaCl_2$

—●— protein value

- -△- - proteolytic activity

Further work on the structural and enzymatic characterization of these trypsin fractions is obviously necessary to verify this suggestion.

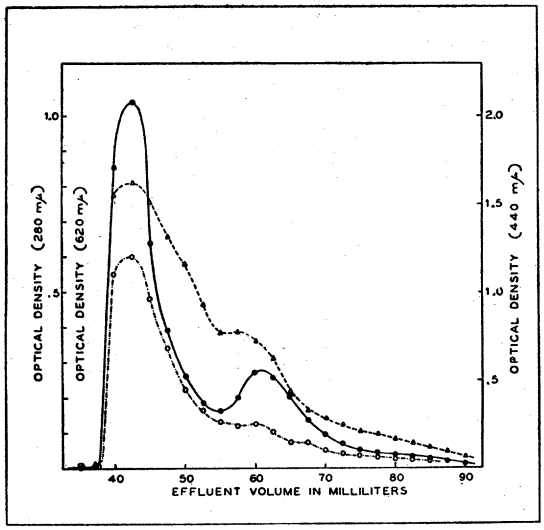


Fig. 4 — Column electrophoresis of the sample of Novo trypsin

—●— protein value
--△-- proteolytic activity
-·-○-·- amidasic activity

## BIBLIOGRAPHY


[1] PERRONE, J. C., DISITZER, L. V. AND DOMONT, G. B. (1959), *Nature*, **183**, 605.

[2] PERRONE, J. C., DISITZER, L. V. AND IACHAN, A., (1959), *Nature*, **184**, 1225.

[3] KUNKEL, H. (1954), In D. Glick. *Methods of Biochemical Analysis*. New York, Interscience Publishers, Inc., I, pg. 160.

[4] CHARNEY, J. AND TOMARELLI, R. M., (1947), *J. Biol. Chem.*, **171**, 501.

[5] PERRONE, J. C., DISITZER, L. V., DOMONT, G. B. AND IACHAN, A., Unpublished experiments.

[6] LIENER, I. E., (1960), *Arch. Biochem. Biophys.*, **88**, 216.

[7] DAVIE, E. W. AND NEURATH, H., (1955), *J. Biol. Chem.*, **212**, 515.

[8] GLADNER, J. A. AND FOLK, J. E., (1958), *J. Biol. Chem.*, **231**, 393.